lyzed for [^{3}H]thymidine incorporation into DNA and for total protein content (bicinchoninic acid protein assay; Pierce). We performed the experiment on nine occasions, with $n = 3$ embryos per concentration.
11. To test antimetabolites, the dU suppression tests were performed with CD1 embryos, cultured in the presence of aminopterin (1 mg/ml), 5-fluorouracil (2 mg/ml), or cycloleucine (2 mg/ml) (minimum teratogenic doses established in preliminary studies). These experiments were performed on two to four occasions, with $n = 3$ embryos per group.
12. CD1 embryos were cultured from E8.5 to E9.5 in folate-deficient serum prepared by extensive dialysis of rat serum as described (*20*, *26*) with addition of *myo*-inositol (10 μg/ml). Folic acid (1.0 mg/ml) was added to the control cultures. The experiment was performed on three occasions, with $n = 6$ embryos per group.
13. CD1 random-bred mice (Charles River, UK) served as nonmutant controls. Mutant mice were *splotch* (*Sp^{2H}*), loop-tail (*Lp/+*), and curly tail (*ct*). The *Sp^{2H}* allele was maintained on a mixed C3H/He, 101, and CBA/Ca background. Litters from heterozygote matings were genotyped by using a *Pax3*-specific polymerase chain reaction (PCR) (*22*). *Lp/+* mice were produced by mating congenic LPT/Le congenic males with inbred CBA/Ca females (Harlan Olac, UK). Litters from heterozygote matings were genotyped by *Crp* PCR (*27*). All *curly tail* individuals were homozygous *ct/ct*, of which 45 to 55% exhibited tail defects or open spinal NTDs. Embryos were categorized as affected or unaffected on the basis of posterior neuropore length at the 27- to 29-somite stage (*28*). The dU suppression tests were performed on mutant embryos on four to seven occasions, with $n = 3$ to 6 embryos per group. Animal care was in accordance with UK governmental legislation (project licence 80/00503).
14. *Splotch* and CD1 embryos were cultured from E8.5 to E10.5 in the continuous presence of folic acid (200 μg/ml), thymidine (250 μg/ml), dUMP (500 μM), or methionine (1.5 mg/ml) (maximum nonteratogenic doses established in preliminary studies).
15. Pregnant *splotch* heterozygotes were injected intraperitoneally on E8.5 and E9.5 with folic acid or thymidine (each at 10 mg/kg body weight) or with an equivalent volume of PBS. Litters were collected at E12.5 or E13.5. We recorded the number of viable embryos and resorptions (no significant difference between groups, $P > 0.05$) and scored the embryos for cranial and spinal NTDs without knowledge of the treatment group. Figures of *splotch* embryos demonstrating prevention of NTDs by folic acid and thymidine are at: www.sciencemag.org/feature/data/981046.shl
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p115 RhoGEF, a GTPase Activating Protein for $G\alpha_{12}$ and $G\alpha_{13}$

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Members of the regulators of G protein signaling (RGS) family stimulate the intrinsic guanosine triphosphatase (GTPase) activity of the α subunits of certain heterotrimeric guanine nucleotide–binding proteins (G proteins). The guanine nucleotide exchange factor (GEF) for Rho, p115 RhoGEF, has an amino-terminal region with similarity to RGS proteins. Recombinant p115 RhoGEF and a fusion protein containing the amino terminus of p115 had specific activity as GTPase activating proteins toward the α subunits of the G proteins G_{12} and G_{13}, but not toward members of the G_s, G_i, or G_q subfamilies of Gα proteins. This GEF may act as an intermediary in the regulation of Rho proteins by G_{13} and G_{12}.

G proteins transduce signals from a large number of cell surface heptahelical receptors to various intracellular effectors, including adenylyl cyclases, phospholipases, and ion channels. Each heterotrimeric G protein is composed of a guanine nucleotide–binding α subunit and a high-affinity dimer of β and γ subunits. Gα subunits are commonly grouped into four subfamilies (G_s, G_i, G_q, and G_{12}) on the basis of their amino acid sequences and function (*1*). The G_{12} subfamily has only two members, α_{12} and α_{13} (*2*). $G\alpha_{12}$ and $G\alpha_{13}$ participate in cell transformation and embryonic development, but the signaling pathways that are regulated by these proteins have not been identified (*3*). However, the small GTPase Rho mediates the formation of actin stress fibers and the assembly of focal adhesion complexes induced by the expression of constitutively active forms of $G\alpha_{12}$ or $G\alpha_{13}$ (*4*).

Members of the RGS family of proteins negatively regulate G protein signaling (*5*). The family includes at least 19 members in mammals and is defined by a core domain called the RGS box. Several RGS proteins act as GTPase activating proteins (GAPs) for α subunits in the G_i or G_q subfamilies (*6*, *7*). The crystal structure of a complex between RGS4 and AlF_4^--activated $G\alpha_{i1}$ revealed that the functional core of RGS4 (the RGS box), which is sufficient for GAP activity (*8*), contains nine α helices that fold into two small subdomains (*9*). The residues of the box that form its hydrophobic core are conserved, and they are important for the stability of structure and GAP activity (*9*, *10*). RGS4 stimulates the GTPase activity of $G\alpha_{i1}$ predominantly by interacting with its three mobile switch regions, thereby stabilizing the transition state for GTP hydrolysis (*9*, *11*).

The activities of members of the Rho family of monomeric GTPases are regulated by guanine nucleotide exchange factors (GEFs) that contain a dbl homology (DH) domain (*12*). Examination of the sequence of p115 (*13*), a GEF specific for Rho, reveals an NH_2-terminal region with similarity to the conserved domain of RGS proteins (Fig. 1). Most of the hydrophobic residues that form the core of this domain (17 of 23) are conserved in p115 RhoGEF. The positions of breaks in the alignment correspond to the loops between α helices in the RGS domain structure. This suggests that p115 RhoGEF may have a similar structural domain and GAP activity. However, the residues of RGS4 that make contact with the switch regions of $G\alpha_{i1}$-GDP-AlF_4^- (GDP, guanosine diphosphate) are not well conserved in p115 RhoGEF, sug-

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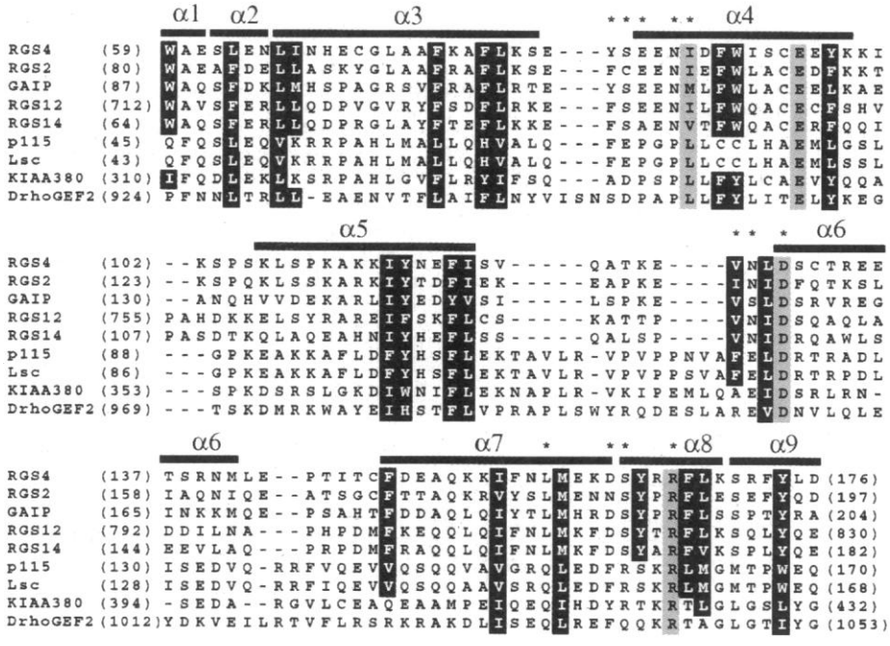
Fig. 1. Sequence alignment of the NH_2-terminal region of p115 RhoGEF with selected RGS proteins. Sequences were aligned with the program Clustal W and secondary structure prediction, based on the structure of RGS4, to assign penalties for gaps. The sequences of Lsc, KIAA380, and DRhoGEF2 were added to this alignment with Clustal W and manual adjustments. The thick lines above the RGS4 sequence indicate the positions of α helices in RGS4 (*9*). Dark shaded boxes indicate conserved residues of the hydrophobic core of the RGS box. Lightly shaded boxes show other conserved residues. Asterisks mark the residues of RGS4 that contact $G\alpha_{i1}$. Primary sequences used in the alignment: rat RGS4 (accession number P49799), mouse RGS2 (O08849), human GAIP (P49795), rat RGS12 (O08774), rat RGS14 (O08773), human p115 RhoGEF (U64105), mouse Lsc (U58203), human KIAA380 (AB002378), and *Drosophila* DRhoGEF2 (AF032870). Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.



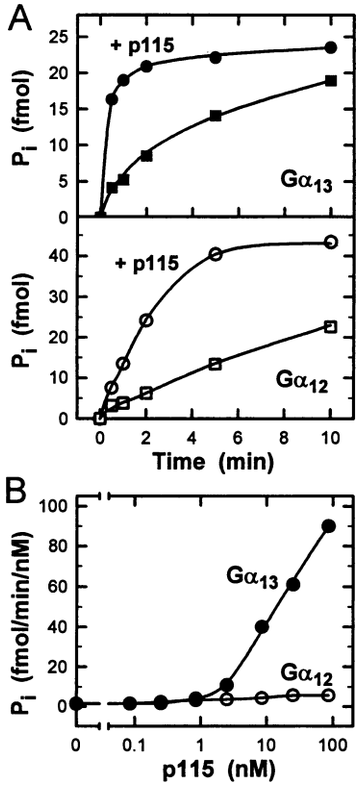


Fig. 2. The p115 RhoGEF protein stimulates GTPase activity of $G\alpha_{13}$ and $G\alpha_{12}$. (**A**) Hydrolysis of GTP bound to $G\alpha_{13}$ and $G\alpha_{12}$ at 15°C either with (circles) or without (squares) 10 nM p115 RhoGEF (*15*). (**B**) Hydrolysis of GTP bound to $G\alpha_{13}$ (●) or $G\alpha_{12}$ (○) was measured at 4°C in the presence of the indicated concentrations of p115 RhoGEF. The initial rates of the reactions are plotted as a function of the concentration of p115 RhoGEF.

gesting differences in the mechanism of action or specificity of p115 RhoGEF relative to other RGS proteins.

The capacity of $G\alpha_{12}$ and $G\alpha_{13}$ to activate Rho in vivo suggested a relationship with p115 RhoGEF, and a physical interaction between p115 and $G\alpha_{13}$ was detected (*14*). Moreover, p115 RhoGEF stimulated hydrolysis of [γ-^{32}P]GTP bound to either $G\alpha_{13}$ or $G\alpha_{12}$ (Fig. 2) (*15*). At 15°C, 10 nM p115 RhoGEF increased the k_{cat}'s for hydrolysis of GTP by $G\alpha_{12}$ (0.07 min^{-1}) and $G\alpha_{13}$ (0.24 min^{-1}) by factors of 5 and 10, respectively (Fig. 2A). Similar results were obtained with several preparations of $G\alpha_{12}$ and $G\alpha_{13}$, and treatment of p115 RhoGEF at 90°C for 5 min inactivated its GAP activity. Because of rapid hydrolysis of GTP by $G\alpha_{13}$, assays were performed at 4°C to estimate more accurately the effect of p115 RhoGEF on the rate of GTP hydrolysis by the G protein (Fig. 2B). Under these conditions, 100 nM p115 RhoGEF increased the GTPase activities of $G\alpha_{13}$ and $G\alpha_{12}$ by factors of 80 and 6, respectively. Thus, although stimulation of the GTPase activity of both proteins was observed at concentrations of p115 RhoGEF as low as 1 nM, p115 RhoGEF was a substantially more efficacious GAP for $G\alpha_{13}$ than for $G\alpha_{12}$.

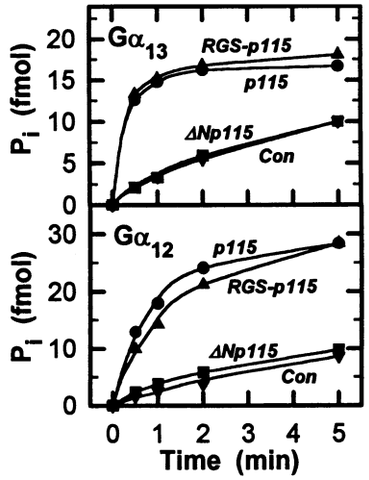


Fig. 3. Stimulation of the GTPase activity of $G\alpha_{13}$ and $G\alpha_{12}$ by the NH_2-terminal region of p115 RhoGEF. Hydrolysis of GTP bound to $G\alpha_{13}$ and $G\alpha_{12}$ was measured at 15°C without (▼) or with (●) 10 nM p115 RhoGEF, 10 nM RGS-p115 (▲), or 50 nM ΔN-p115 RhoGEF (■).

In the absence of a receptor, the rate-limiting step for binding of the nonhydrolyzable GTP analog guanosine 5′-*O*-(3′-thiotriphosphate) (GTP-γ-S) to Gα proteins and for their steady-state hydrolysis of GTP is the release of GDP. The p115 RhoGEF protein did not affect the rate of GTP-γ-S binding to $G\alpha_{12}$ or $G\alpha_{13}$, nor did it affect the steady-state GTPase activity of either subunit (*16*). Therefore, p115 RhoGEF stimulates only the intrinsic GTPase activity of $G\alpha_{12}$ and $G\alpha_{13}$ without affecting their rates of nucleotide exchange.

The conserved RGS box of RGS proteins is sufficient for GAP activity in vitro (*8*). We thus tested a fusion protein (*17*) containing glutathione S-transferase (GST) and the NH_2-terminal region of p115 RhoGEF for GAP activity. The fusion protein, which contains the RGS similarity region (Fig. 1) but not the DH or PH domains of p115 RhoGEF, was almost as active as full-length p115 (Fig. 3). In contrast, p115 RhoGEF

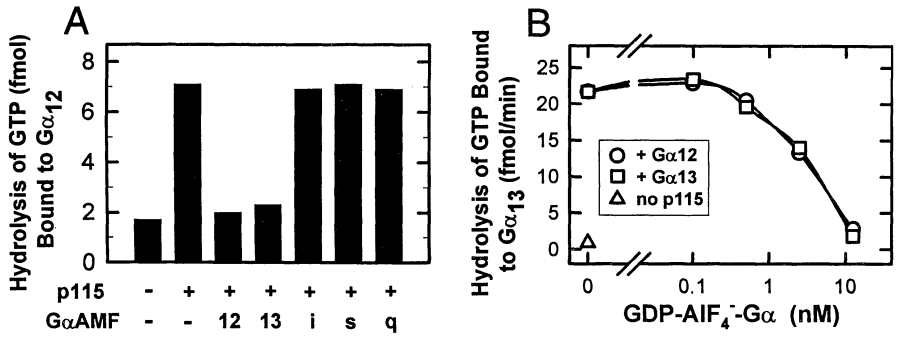


Fig. 4. Selective inhibition of p115 RhoGEF GAP activity by AlF_4^--activated Gα subunits. (**A**) p115 RhoGEF (400 nM) was incubated on ice for 15 min with various Gα subunits (400 nM) in the presence of AMF (30 μM $AlCl_3$, 10 mM NaF, and 10 mM $MgSO_4$). The mixtures were diluted with 19 volumes of buffer A (*15*) and mixed with 0.3 nM GTP-$G\alpha_{12}$. The hydrolysis of bound GTP was measured after incubation at 15°C for 2 min in the presence of AMF. (**B**) p115 RhoGEF (400 nM) was incubated with various concentrations of GDP-AlF_4^--$G\alpha_{12}$ (○) or GDP-AlF_4^--$G\alpha_{13}$ (□) as in (A). The mixtures were diluted with 19 volumes of buffer A and mixed with 1 nM GTP-$G\alpha_{13}$; the hydrolysis of bound GTP was assessed over time at 4°C in the presence of AMF. The initial rate of GTP hydrolysis by $G\alpha_{13}$ was plotted against the final concentration of GDP-AlF_4^-–bound α subunit (△ indicates the rate of GTP hydrolysis by $G\alpha_{13}$ in the absence of p115 RhoGEF).

truncated at the NH_2-terminus to eliminate the RGS box was ineffective. Thus, the RGS homology region mediated the GAP activity of p115 RhoGEF.

The p115 RhoGEF protein (100 nM) did not stimulate the GTPase activity of $G\alpha_{i1}$, $G\alpha_z$, or $G\alpha_q$ under conditions where RGS4 did act as a GAP for these Gα subunits (*16*). Similarly, p115 RhoGEF did not accelerate the GTPase activity of $G\alpha_s$, RhoA, or Rac1 (*16*). Thus, p115 RhoGEF is a GAP with specificity for $G\alpha_{12}$ and $G\alpha_{13}$. However, it is possible that this specificity could be different in the presence of an activated receptor. The GAP activity of RGS2 toward $G\alpha_{i1}$ was observed only after reconstitution of the proteins into phospholipid vesicles containing M2 muscarinic cholinergic receptors (*18*).

RGS proteins have relatively high affinity for the GDP-AlF_4^-–bound forms of G protein α subunits, whose conformation is similar to that of the transition state for GTP hydrolysis (*11*). Therefore, the GDP-AlF_4^-–bound forms of appropriate Gα proteins should compete with Gα-GTP for interaction with p115 RhoGEF. GDP-AlF_4^-–bound $G\alpha_{12}$ and $G\alpha_{13}$ inhibited GAP activity of p115 RhoGEF on $G\alpha_{12}$, whereas similar forms of $G\alpha_s$, $G\alpha_{i1}$, and $G\alpha_q$ did not (Fig. 4A); these results are indicative of the selectivity of p115 RhoGEF. Furthermore, the GDP-AlF_4^-–bound forms of $G\alpha_{12}$ and $G\alpha_{13}$ are equipotent inhibitors of the GAP activity of p115 RhoGEF toward $G\alpha_{13}$ (Fig. 4B).

Although we assume that the structure of the GAP domain of p115 RhoGEF is similar to that of the RGS box of RGS4 and that it interacts with the switches of $G\alpha_{12}$ and $G\alpha_{13}$, the amino acid sequences of these switch regions differ between the G_{12} and G_i subfamilies (*1*, *2*). Six of the seven residues in RGS4 that interact with Thr^{182} of $G\alpha_{i1}$, a residue of switch I that is in the center of the RGS4-$G\alpha_{i1}$ interface (*9*), are not conserved in p115 RhoGEF. Thus, the surface between p115 and $G\alpha_{12}$ or $G\alpha_{13}$ is likely quite different from that observed with RGS4 and $G\alpha_{i1}$, and this amino acid diversity must contribute to the different specificity of the GAP activity of p115 RhoGEF relative to other members of the RGS family.

Three other RhoGEF proteins (Lsc, KIAA380, and DRhoGEF2) contain regions of similarity to the RGS domain of p115 RhoGEF (Fig. 1). Lsc appears to be the mouse homolog of p115 RhoGEF, and KIAA380 is likely the human homolog of *Drosophila* DRhoGEF2 (*19*, *20*). The latter provides a biological correlate for the biochemical relationships defined for $G\alpha_{13}$ and p115 RhoGEF. DRhoGEF2 is a mediator of a signal critical for gastrulation (*20*), and genetic evidence suggests that DRhoGEF2 functions downstream of a G protein α subunit, concertina (Cta), that is most similar to the mammalian $G\alpha_{12}$ and $G\alpha_{13}$ (*21*). The four RhoGEFs may define a new subset of RGS proteins that not only have GAP activities but also couple RhoGEF activity to G protein α subunits. This coupling was observed in the stimulation of the exchange activity of p115 RhoGEF by Ga_{13} (*14*).

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16. T. Kozasa and P. M. Sternweis, unpublished data.
17. Recombinant p115 RhoGEF and ΔN-p115 RhoGEF contain NH_2-terminal Glu-tags and were expressed in Sf9 cells using recombinant baculoviruses and affinity-purified as described (*13*). A fusion (RGS-p115) of GST to the first 246 amino acids of p115 RhoGEF was constructed in pGEX4T-2 (Pharmacia), expressed as a chimeric protein in *Escherichia coli*, and purified by glutathione-Sepharose affinity chromatography.
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